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Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells

(*Bacillus anthracis*/cholera toxin/Chinese hamster ovary cells)

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ABSTRACT **Anthrax toxin is composed of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). These proteins individually cause no known physiological effects in animals but in pairs produce two toxic actions. Injection of PA with LF causes death of rats in 60 min, whereas PA with EF causes edema in the skin of rabbits and guinea pigs. The mechanisms of action of these proteins have not been determined. It is shown here that EF is an adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] produced by *Bacillus anthracis* in an inactive form. Activation occurs upon contact with a heat-stable eukaryotic cell material. The specific activity of the resulting adenylate cyclase nearly equals that of the most active known cyclase. In Chinese hamster ovary cells exposed to PA and EF, cAMP concentrations increase without a lag to values about 200-fold above normal, remain high in the continued presence of toxin, and decrease rapidly after its removal. The increase in cAMP is completely blocked by excess LF. It is suggested that PA interacts with cells to form a receptor system by which EF and perhaps LF gain access to the cytoplasm.**

Evidence that the virulence of *Bacillus anthracis* is due in part to a protein exotoxin was first obtained in 1955 by Smith *et al.* (1) when they found that plasma from guinea pigs dying of anthrax produced edema in the skin of normal guinea pigs and was lethal to mice. The crude toxin subsequently was produced in broth cultures and resolved into three components, which American workers (2, 3) designated edema factor (EF), protective antigen (PA), and lethal factor (LF). British workers call these materials factors I, II, and III, respectively (4, 5). The individual toxin components have no known biological effects when administered alone, but EF injected with PA into the skin of rabbits or guinea pigs causes edema, and PA injected with LF into rats causes death in as little as 60 min. Experiments involving sequential injections of PA and LF led to the hypothesis that the factor common to both activities, PA, binds to tissue receptors and permits subsequent action of either LF or EF (6). This model was supported by the demonstration that LF blocks the action of EF (4).

Procedures were developed for the purification of PA (7), which currently is used as a vaccine to protect laboratory personnel, veterinarians, and certain industrial workers who might contact anthrax spores. LF and EF were purified partially but in amounts too small to permit determination of their mechanisms of action (8, 9). Tests on partially purified components failed to detect any of 12 different enzymatic activities (5), and *B. anthracis* culture supernatants had no toxic effect on three established eukaryotic cell lines (10). No further studies on the mechanism of action of anthrax toxin are known to have been performed since 1967. Recent work in this laboratory has led to the preparation of several milligrams of LF and EF at purities of 80%. It is shown here that EF is an adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] that acts in the cytoplasm of eukaryotic cells, causing dramatic elevations in cAMP concentrations. Therefore, EF appears to be the archetype of a novel category of bacterial toxins.

MATERIALS AND METHODS

Toxins. *B. anthracis* Sterne strain was obtained from Anna Johnson-Winegar (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD). This noncapsulated, avirulent strain produces all three components of anthrax toxin (11). Similar strains are used as live veterinary vaccines. PA produced by growth of this strain in a defined medium (7) was purified by sequential chromatography on DEAE-cellulose (12) and hydroxylapatite (unpublished data). Fractions containing PA were identified by immunodiffusion against a burro antiserum raised by repeated injection of Sterne strain spores (gift of Anna Johnson-Winegar). A 20-liter-fermenter culture yielded 30 mg of purified PA. Electrophoresis on polyacrylamide gels in the presence of $NaDodSO_4$ showed that the PA used was more than 90% pure and consisted of a polypeptide of M_r ≈80,000. LF and EF were produced by growth of the Sterne strain in a Casamino acids medium (13) and purified by sequential DEAE-cellulose (12) and hydroxylapatite chromatography. LF was located by injection with purified PA into Fisher 344 rats (13). EF was located by adding diluted column fractions to microtiter plates containing 1 μg of PA per ml and Chinese hamster ovary (CHO) cells and by scoring the cells for visible elongation. A 20-liter fermenter yielded 5 mg of LF and 2 mg of EF. These two proteins were each ≈80% pure as judged by electrophoresis in $NaDodSO_4$, and both also had a M_r ≈80,000. Cholera toxin (CT) was obtained from List Biological Laboratories, Campbell, CA.

Cell Culture Methods. A CHO cell variant designated CHO K1A was obtained from Michael Gottesman, National Institutes of Health. These cells were maintained as monolayer cultures in Eagle's minimal essential medium supplemented with nonessential amino acids, further supplemented with 25 mM Hepes (pH 7.4), gentamicin (50 μg/ml), and 5% heat-inactivated fetal calf serum. Baby hamster kidney (BHK)-21 cells (ATCC CCL 10) were obtained from the American Type Culture Collection and maintained in the recommended medium. Fetal rhesus lung (FRL)-103 cells (also designated FRhL-2, DBS-103) were obtained from the Salk Institute and maintained in Eagle's minimal essential medium supplemented with nonessential amino

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Abbreviations: EF, edema factor; PA, protective antigen; LF, lethal factor; CT, cholera toxin; CHO, Chinese hamster ovary; PEI, poly ethyleneimine; BHK, baby hamster kidney; FRL, fetal rhesus lung.

acids and 10% fetal calf serum. Established cell monolayers in 24-well tissue culture dishes were exposed to toxins at 37°C in medium 199 containing Hanks' salts (H199), 25 mM Hepes (pH 7.4), and either fetal calf serum or bovine serum albumin. To measure cellular cAMP content, toxin-treated monolayers were washed twice with cold Hanks' balanced salt solution and extracted for 20–60 min with 250 μl of 0.1 M HCl. cAMP was measured by radioimmunoassay (14) using the kit from New England Nuclear. Residual cell protein was dissolved in 0.1 M NaOH and assayed by using an automated Lowry procedure (15). To prepare the CHO cell lysate used in the cyclase assays, cells scraped from two 75-cm^2 flasks were swollen in 1.0 ml of hypotonic buffer (25 mM Hepes, pH 7.4/1 mM dithiothreitol/1 mM EDTA) and lysed by sonication (16). Nuclei and unbroken cells were removed by centrifugation at 900 × g for 10 min. The lysate contained 1.4 mg of protein per ml. A 100-μl portion of the lysate was heated at 95°C for 3 min to give "boiled lysate."

Adenylate Cyclase Assay. Reaction mixtures of 50 μl contained 50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.50 mM EDTA, 0.70 mM dithiothreitol, 0.50 mM 3-isobutyl-1-methylxanthine, 200 μg of bovine serum albumin per ml, 13 or 270 ng of EF, and 0.50 mM ATP (Sigma A-2383) that contained $\approx 2 \times 10^5$ dpm (3.3×10^3 becquerels) of [α-^{32}P]ATP (New England Nuclear, NEG-003). Reactions were terminated by addition of 100 μl of 0.1 M HCl and application of heat to boiling. After dilution to 1.0 ml with 0.10 M imidazole chloride buffer (pH 7.0), the samples were passed through 1.0-ml alumina columns (17), followed by 6 ml of the same buffer. The total eluate of each column was assayed for radioactivity by using Cerenkov radiation.

Thin-Layer Chromatography. Adenylate cyclase reactions (50 μl) were stopped by addition of 100 μl of 0.40 M perchloric acid. A mixture of nonradioactive nucleotides was added, and the samples were neutralized with 50 μl of 0.80 M KOH. The precipitates were removed, and a portion of each supernatant was spotted on a polyethyleneimine (PEI)-cellulose plate (Baker-Flex type PEI-F). The chromatogram was developed by placing it consecutively into four eluents so that the liquid front traveled 3, 3, 3, and 6 cm while the plate was in solvents containing 0.3, 0.9, 1.5, and 1.9 M LiCl, respectively. All four solvents also contained 2.5% acetic acid and 30% ethanol. Marker nucleotides were located under an ultraviolet lamp, and ^{32}P-labeled materials were located by autoradiography with DuPont Cronex 4 film.

RESULTS

My efforts to determine the mechanism of action of anthrax toxin began with the recognition that the edematous response to EF and PA resembled that caused by cholera toxin (CT). The latter toxin is frequently assayed in rabbit skin, where it causes increased vascular permeability (18). This similarity suggested that EF, like CT, might act by elevating intracellular concentrations of cAMP. A convenient test for elevation of cAMP concentrations exploits the fact that certain cultured cells respond to CT with a characteristic shape change (19). A CHO cell variant (CHO K1A), selected as showing a particularly well-defined shape change, was obtained from Michael Gottesman. CHO K1A cells exposed to supernatants of Sterne strain broth cultures did show the same characteristic morphological change as seen in parallel CT-treated cells. Thus, isolated cells became substantially elongated within 2 hr and appeared more adherent to the plastic substrate. This morphological response then was used to locate the active EF material during its purification by chromatography on DEAE-cellulose and hydroxylapatite. The purified material obtained in this way caused CHO cell elongation only when mixed with PA, and the elongation response was blocked by LF. Although it has not yet been tested for edema formation, the behavior of the purified material in the CHO assay shows it to be functionally identical to the EF characterized by previous workers. The EF preparation used here contains a M_r 80,000 polypeptide contributing about 80% of the total material. Although it seems likely that this protein (rather than the other minor peptides present) possesses the activities described below, this has not been proven; therefore, the term EF must be viewed as describing a functional and not a physical entity.

The hypothesis that the characteristic shape change caused by PA and EF reflected increased cAMP concentrations was found to be correct when direct measurements of cAMP were made (Fig. 1). With PA held constant at 1 μg/ml, EF added at concentrations as low as 10 ng/ml caused significant cAMP increases, whereas addition of EF at 1 μg/ml caused increases exceeding 200-fold. Addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine caused an additional 2-fold increase. In these experiments, the cAMP extracted from cell monolayers was acetylated and measured by competitive radioimmunoassay (14). Similar values were obtained when extracts were assayed without acetylation.

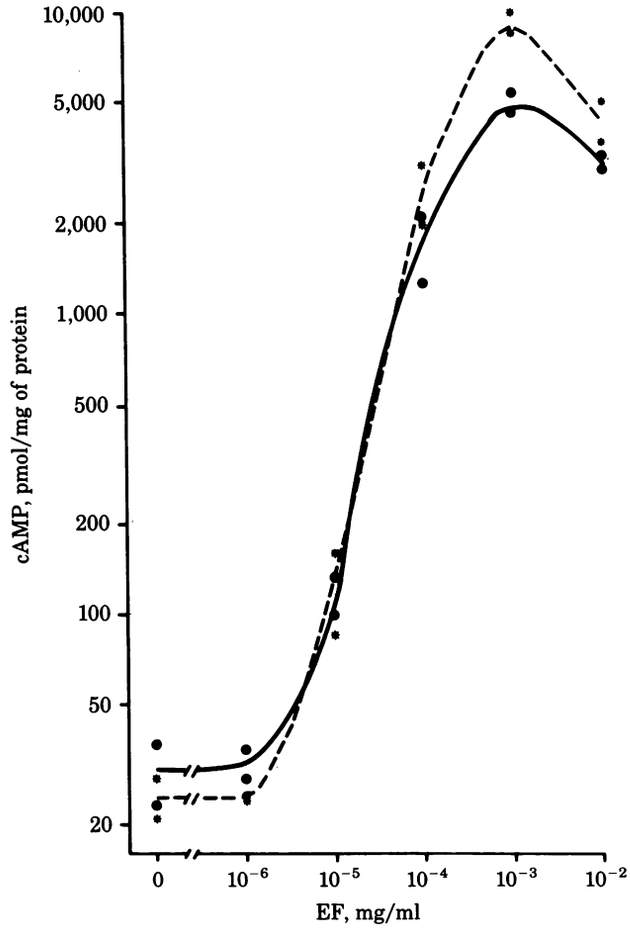

FIG. 1. cAMP response of CHO cells to anthrax toxin: dependence on EF concentration. CHO cells were plated in 24-well tissue culture dishes and grown to confluency. To begin the experiment, the medium was replaced with warm H199 medium containing 25 mM Hepes, 1% fetal calf serum, and 1 μg of PA per ml. Half of the wells received the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at 0.5 mM. EF was added to duplicate wells at the indicated concentrations. After incubation for 2 hr at 37°C, the monolayers were washed and extracted with 0.1 M HCl, and cAMP was assayed. Residual precipitated cell protein was dissolved in 0.1 M NaOH and measured by an automated Lowry method. Each point is the result of a single assay on a separate well. ●, EF; *, EF with 0.5 mM 3-isobutyl-1-methylxanthine.

Dose–response analyses to determine the optimum PA concentration were performed with EF at several concentrations. At each EF concentration, the cAMP response reached a plateau at 1 μg of PA per ml (data not shown). The heights of the plateaus increased with the fixed EF concentration up to about 1 μg of EF per ml. Therefore, it appears that the cellular systems on which PA and EF act become saturated at 1 μg of each component per ml. The elevation in cAMP required the presence of both PA and EF (Table 1). Addition of either component alone at 1 μg/ml did not increase cAMP concentrations above control values, whereas the combination caused an increase exceeding 50-fold. This result also shows that the PA and EF samples are free of cross-contamination. Sensitivity to the combination of PA and EF is not a unique feature of CHO cells. Thus, the two additional cell lines tested in the experiment of Table 1, BHK-21 and FRL-103, behaved in essentially the same manner as did CHO cells. Preliminary experiments indicate that many cultured cells show at least some increase in cAMP when treated with PA and EF.

As a further test of whether the actions seen in cultured cells parallel those described in whole animals (4), the ability of LF to block the action of EF was measured (Table 1). In each of the three cell lines, addition of a 20-fold weight excess of LF over EF abolished the cAMP response. LF alone or with PA did not increase cAMP concentrations (not shown).

CT elevates cAMP concentrations by ADP-ribosylating and, thereby, permanently activating a subunit of eukaryotic cell adenylate cyclase (20, 21). To determine whether the combination of PA with EF might act through a similar mechanism, the general properties of the CHO cell response to these toxins were compared (Fig. 2). In cells treated with CT, cAMP concentrations increased only after a delay of 30 min and did not reach a plateau until 90 min. The initial lag is a consistent feature of the response of intact cells to CT (22). In contrast, the cells exposed to PA and EF showed a rapid increase in cAMP concentrations, with no indication of a lag. Near maximal values were reached after 60 min of toxin treatment; these exceeded by 10-fold the concentrations produced by CT. Because CT action involves a covalent modification of cyclase that is effectively irreversible, washing of cells to remove unbound toxin does not lead to a decrease in cAMP levels (Fig. 2*B*). However, cells treated with PA and EF and subsequently washed showed a significant decrease. These results suggested that the anthrax toxin might not act, like CT, by ADP-ribosylation of cyclase.

Table 1. Cellular cAMP contents of cultured cell lines treated with combinations of anthrax toxin components

Toxin components, μg/ml			cAMP in cell lines, nmol/mg		
PA	EF	LF	CHO	BHK-21	FRL-103
—	—	—	0.100	0.065	0.071
1.0	—	—	0.081	0.075	0.075
—	1.0	—	0.087	0.075	0.075
1.0	1.0	—	5.7	3.1	13
1.0	0.05	—	1.4	0.52	3.4
1.0	0.05	0.1	0.73	0.18	1.1
1.0	0.05	1.0	0.16	0.048	0.082
1.0	0.05	10.0	0.079	0.040	0.075

Cells plated in 24-well tissue culture dishes were treated in H199 medium containing 25 mM Hepes and 5% fetal calf serum for 2.5 hr with the indicated toxin components. The monolayers were washed, extracted with 0.1 M HCl, and aliquots of the extracts were assayed for cAMP. A single determination was done on the extract from each of three identically treated wells. Values are means for the three determinations. SEMs ranged up to 50% for means below 0.1 and up to 15% for means above 0.1. Protein was assayed as in Fig. 1. Protein contents of wells averaged 48, 60, and 28 μg for CHO, BHK-21, and FRL-103, respectively.

An alternate explanation for the action of anthrax toxin is that PA and EF individually or in combination constitute an adenylate cyclase capable of acting in the cytoplasm of eukaryotic cells. Table 2 shows that EF expresses a strong adenylate cyclase activity but only when a CHO cell lysate is present. Because the activity is also present when a boiled lysate is used, and because the cyclase activity is more than 100-fold greater than that expected from the CHO cell cyclase, it can be concluded that EF is contributing the catalytic entity. Therefore, EF is an adenylate cyclase that, like several other bacterial toxins, is synthesized in an inactive form. Activation requires the presence of a heat-stable eukaryotic cell material. Preliminary evidence indicates that calmodulin may be this activator. Thus, activity was obtained when CHO cell lysate was replaced by a purified calmodulin sample (gift of Carol Linden, this institute), and activity was inhibited by calcium chelators and by chlorpromazine.

In order to confirm the identification of EF as an adenylate cyclase, the reaction product was examined by a second separation technique. Reactions like those in Table 2 were chromatographed on PEI-cellulose thin-layer plates, which were then exposed to x-ray film. Nearly all of the [α-^{32}P]ATP was converted to a band that coincides exactly with marker, nonradioactive cAMP (Fig. 3).

DISCUSSION

The recognition that EF is an adenylate cyclase suggests a plausible model for its action that is consistent with the experiments reported here and with the extensive studies of anthrax toxin in whole animals. This model envisions the existence of specific cell surface receptors for PA. Binding of PA to these receptors produces a new type of receptor that is recognized by both EF and LF. EF bound at this site is inserted into or transferred across the membrane so as to gain functional access to the cytoplasm. Interaction with a heat-stable substance, probably calmodulin, generates an efficient adenylate cyclase. The resulting increases in cAMP cause the edematous response in skin and presumably other effects in the tissues of infected animals. The existence of cell surface receptors for PA was originally inferred from the need for PA in both the edema and lethal responses. The demonstration that EF is an adenylate cyclase shows that this component must function in the cytoplasm, the location of its substrate and product. Because EF action on intact cells absolutely requires PA, it follows that PA probably acts at the cell membrane. Binding of PA on the cell surface to form a receptor also provides a consistent explanation for the competitive action of LF and EF, first seen in whole animals and demonstrated here in CHO cells. The fact that dose–response curves for both PA and EF plateau above 1 μg/ml is also consistent with a receptor-mediated process.

Two other bacterial toxins that act by increasing cellular cAMP concentrations have been well characterized. CT and *Escherichia coli* heat-labile enterotoxin bind to ganglioside G_{M1} cell surface receptors and enter the cytoplasm by a mechanism that seems to involve membrane penetration (20–22). Proteolytic activation and disulfide bond reduction of CT free the M_r 21,000 A_1 peptide, which is an ADP-ribosyl transferase. Ribosylation of the nucleotide regulatory subunit of adenylate cyclase converts it to a permanently active form. At least one of these steps occurs at a slow rate because a lag is invariably seen between CT addition and the initial rise in cAMP content (22).

EF produces increases in cellular cAMP by a mechanism different from that of CT. Therefore, the response of cells to EF differs in many respects from that seen in CT-treated cells. The increase in cAMP concentrations induced by EF appears to proceed without a lag, suggesting that entry into the cell is rapid, perhaps involving direct penetration of the plasma mem-

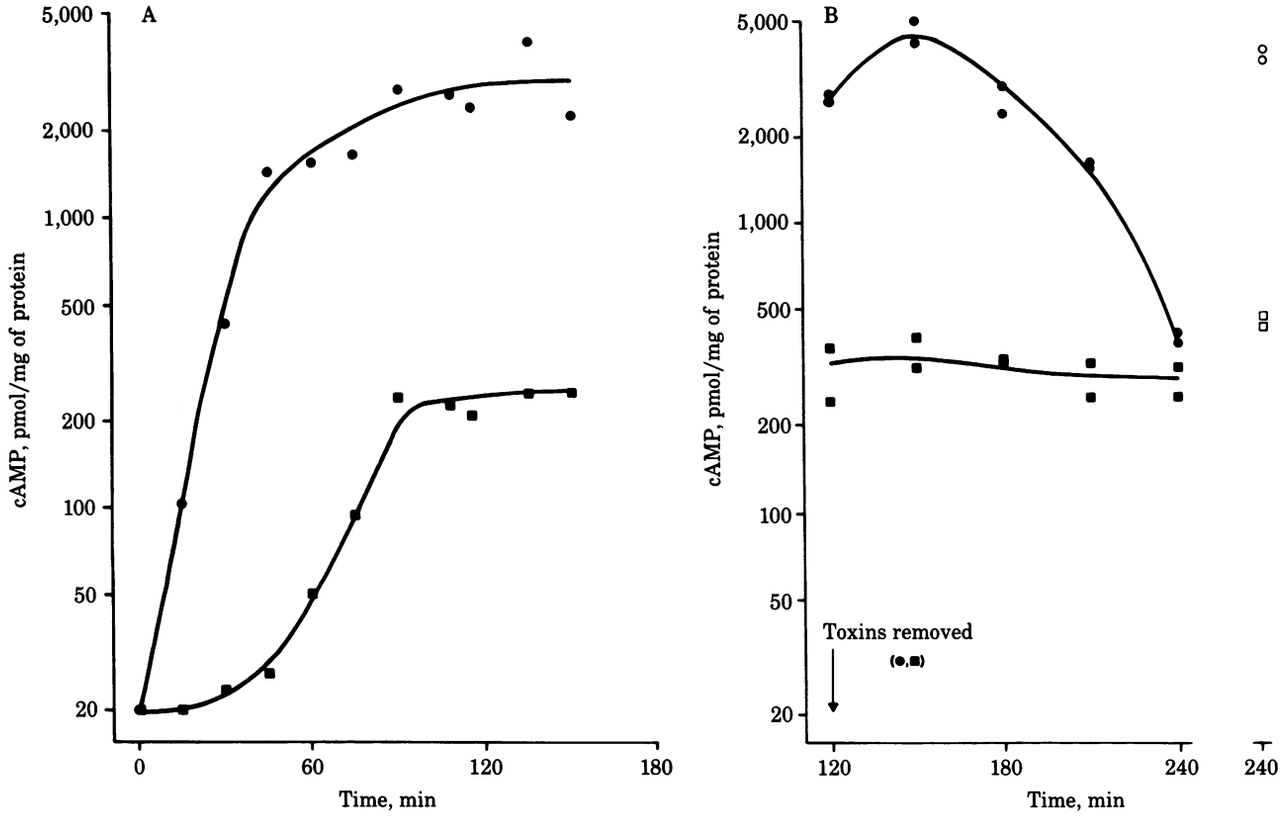

FIG. 2. Kinetics and reversibility of cAMP response in CHO cells treated with anthrax and cholera toxins. (*A*) CHO cells plated in 24-well tissue culture dishes were washed and incubated at 37°C in H199 medium containing 25 mM Hepes and bovine serum albumin (1 mg/ml). PA and EF (●) or CT (■), each at 1 μg/ml, were added for the indicated time. Monolayers were then assayed for cAMP as in Fig. 1. Each point shown is the result of a single assay from a separate well. (*B*) Cells treated with toxins for 120 min as in *A* were washed twice with Hanks' balanced salt solution containing bovine serum albumin (1 mg/ml) and then were placed in fresh toxin-free H199 medium containing bovine serum albumin (1 mg/ml) (●, ■). Duplicate wells were harvested at 30-min intervals and assayed for cAMP. Control wells (○, □) were not washed at 120 min and, therefore, were exposed to toxin continuously for 240 min. Each point shown is the result of a single assay from a separate well.

brane. Activation of the proenzyme must also be rapid, occurring either coincident with the entry event or upon contact with the cytoplasm. Although it seems clear that EF acts on cytoplasmic ATP, no conclusion can be drawn as to what physical event allows this interaction. Treatment of CHO cells with optimal concentrations of PA and EF produces cAMP increases of close to 200-fold, well above the maximal values seen in CT-treated cells. The ability of PA and EF to produce higher concentrations of cAMP is consistent with the fact that the rate of synthesis is not limited by the amount of endogenous adenylate cyclase and suggests that the uptake mechanism that delivers EF to the cytoplasm is quite efficient. No direct evidence is yet available to define the nature of the association of EF with cells. The rapid decrease in cAMP after toxin removal (Fig. 2*B*) suggests either that internalized EF is rapidly degraded or that the association of EF with cells, allowing it to act in the cytoplasm, is readily reversible. The ability of cells to maintain high concentrations of cAMP for up to 4 hr (Fig. 2*B*) indicates that essential components of the uptake system for EF are not consumed during EF action.

Table 2. Adenylate cyclase activity of anthrax toxin components

Toxin components, ng		CHO cell lysate, μg of protein		cAMP formed	
EF	PA	Native	Boiled	Total cpm	Net pmol
—	—	—	—	225	0
270	—	—	—	295	0
—	550	—	—	180	0
270	550	—	—	240	0
—	—	14	—	165	0
—	—	—	14	200	0
13	—	14	—	16,100	3,800
13	—	—	14	11,800	2,800
13	550	—	14	11,900	2,800

Reaction mixtures contained the materials listed here and the buffer and substrate components listed in *Materials and Methods*, in a total volume of 50 μl. Each reaction mixture contained 25 nmol of ATP and 104,000 cpm of [α-^{32}P]ATP. After incubation for 10 min at 37°C, the reactions were stopped with 0.1 M HCl and the mixtures were chromatographed on alumina columns. Assays using lysates as the activator are linear with both time and EF concentration. Values are averages of duplicate determinations, none of which differed by more than 15%. The total, uncorrected cpm in the column effluents are shown, and calculated values of net pmol cAMP are uncorrected for recovery from the columns (usually exceeds 80%) and are, therefore, minimum estimates.

Adenylate cyclases previously have been identified in, and partially purified from, extracts of a number of bacterial species including *E. coli* (23–26), but little attention appears to have been given to their possible involvement in bacterial virulence. Adenylate cyclase activity was not detected in three *Bacillus* species (*cereus*, *pumilus*, *subtilis*; ref. 24) and has not been identified until EF in an isolate from a *Bacillus*. Bacterial adenylate cyclases were initially classified into two groups—those found in the soluble fraction and dependent on pyruvate for activity and those in the particulate fraction and not dependent on pyruvate (24). More recently, an adenylate cyclase purified from *Bordetella pertussis*, the causative organism of whooping cough, was shown to require calmodulin and possibly another protein for activity (25, 27). The *B. pertussis*, *E. coli* (23), and

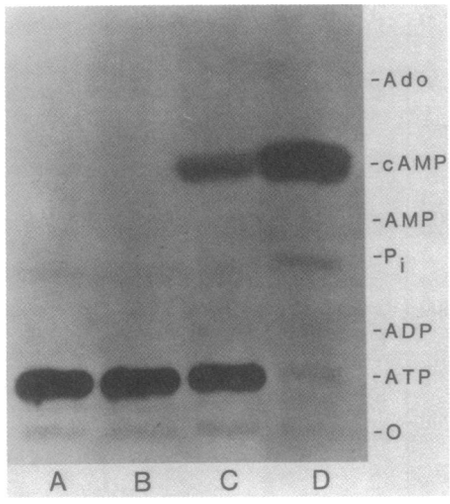

FIG. 3. Thin-layer chromatography of adenylate cyclase reaction mixtures. Reaction mixtures contained the components listed in *Materials and Methods* and boiled CHO cell lysate and EF as noted below, in a total volume of 50 μl. Tubes were warmed 3 min at 37°C, ATP was added, incubation was continued at 37°C for the times noted, and reactions were stopped with 100 μl of 0.40 M perchloric acid. A mixture containing 50 nmol each of nonradioactive ATP, ADP, AMP, cAMP, and adenosine (Ado) was added, and the samples were neutralized with 50 μl of 0.80 M KOH. A 20-μl portion of each supernatant was chromatographed on PEI-cellulose. Marker nucleotides were located under an ultraviolet lamp, and the position of P_i was determined from a sample of $^{32}P_i$ run in an adjacent lane not shown in the figure. Lanes: A, 14 μg of lysate, 20 min; B, 270 ng of EF, 20 min; C, 14 μg of lysate and 270 ng of EF, 1 min; D, 14 μg of lysate and 270 ng of EF, 20 min.

Brevibacterium liquifaciens (26) enzymes have been obtained in a relatively pure state. Of these three enzymes, only the last, obtained in a crystalline form (26), has a specific activity (30 μmol min^{-1} mg^{-1}) comparable to that calculated for EF from the data of Table 2 (20 μmol min^{-1} mg^{-1}). This suggests that EF was obtained in a highly purified state.

Previous work has not demonstrated that EF contributes to the virulence of *B. anthracis*. The dramatic cAMP increases in cultured cells demonstrated here suggest that profound effects might be expected in toxin-treated or infected animals. However, the few thorough histological and physiological studies of infected animals do not clearly point to a cAMP-mediated response (28, 29). Most investigators consider that the pathologic changes seen in infected animals are due to the lethal toxin, composed of PA and LF. These acute effects might be expected to mask any cAMP-mediated responses. In the only studies directly implicating EF as a virulence factor, mice were found to be killed by lower doses of the lethal toxin (PA and LF) when EF was administered simultaneously (4).

If EF does contribute to the virulence of *B. anthracis*, this will occur through the toxin's ability to elevate cAMP concentrations. All of the effects of cAMP seem to be mediated through its activation of cAMP-dependent protein kinase (30, 31). Because this kinase is fully activated at cAMP concentrations that are 5–10 times more than normal values, cAMP increases beyond this value may not have any additional effect on cyclic nucleotide-controlled processes. Therefore, the effects of EF and PA on cells and animals might closely resemble those caused by CT (unless PA receptors are localized to particular tissues). Cell culture studies show CT to have relatively little acute toxicity. This toxin causes growth inhibition in only a few types of cultured cells. However, mice are killed several days after intravenous injection of CT, a median lethal dose being about 2 μg (100 μg/kg of body weight; ref. 32). Although these studies provide some guidance as to the possible physiological effects of EF, the responses of particular tissues and animals remain to be determined.

The work described here identifies a new mechanism by which bacterial proteins may damage animal tissues. The fact that CT and the *E. coli* enterotoxin also act through cAMP suggests that perturbation of this system may be of particular advantage to pathogens. Therefore, a survey of other pathogens for adenylate cyclase activity may identify additional toxins that act in the same manner as EF. If the preliminary evidence identifying calmodulin as the heat-stable activator of EF is confirmed, then the calmodulin-dependent adenylate cyclase of *B. pertussis* merits reexamination as a potential virulence factor.

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